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Whole genome analysis and functional characterization of a novel *Bacillus thuringiensis* (*Bt* 62) isolate against sugarcane white grub *Holotrichia serrata* (F)

Naveenarani M. [a,1], Suresha G.S. [a,1], Srikanth J. [a], Hari K. [a], Sankaranarayanan C. [a], Mahesh P. [a], Nirmala R. [a], Swathik C.P. [a], Crickmore N. [b], Bakshi Ram [a], Appunu C. [a], Singaravelu B. [a,*]

[a] ICAR- Sugarcane Breeding Institute, Coimbatore, Tamil Nadu 641007, India
[b] School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

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ABSTRACT

In this study, we report the whole genome assembly of *Bt* 62, a novel isolate harbouring *cry8* holotype gene identified by us earlier. Sequencing was carried out using a combination of Illumina NextSeq 500 and Oxford Nanopore sequencing Technologies (ONT). The final assembled genome was 6.13 Mb comprising a circular chromosome and four plasmids. The bioassay studies against *Holotrichia serrata* (F.) (Coleoptera: Scarabaeidae), a polyphagous pest infesting sugarcane and other crops, indicated significant toxicity to first instar grubs over untreated larvae achieving a highest mean mortality of 91.11% for various doses tested. *In vitro* proteolytic assay and histopathological studies of the midgut of infected white grubs revealed proteolytic processing of the protoxin and extensive degeneration of larval midgut epithelial cells. The results demonstrate that this novel isolate could be used as a biopesticide or its crystal toxin genes could be expressed in sugarcane and other crops for resistance against *H. serrata.*

1. Introduction

Bacillus thuringiensis, a common soil-dwelling gram positive bacterium, produces crystal proteins that have been found to be toxic to several insect pests belonging to various orders. There are mainly two groups of toxins found in crystals namely, Cry and Cyt, but there are others as well that were renamed newly in 2020 by the *Bt* toxin nomenclature committee [1]. The larvae of many insects are susceptible to the proteins encoded by different *cry* genes [2]. Although several *cry* gene families are found in the Bacterial Pesticidal Protein Resource Centre (https://bpprc.org/), a review by Wang et al. [3]. lists Cry3, Cry8, Cry18, Cry23/37 (now MPP23/XPP37), Cry43, vegetative insecticidal protein families Vip1/Vip2, Cry18 from *Bacillus popilliae* and Cry43 from *Paenibacillus lentimorbus*, as toxic to grubs of beetles belonging to the family Scarabaeidae. The polyphagous white grub *Holotrichia serrata* (F) is one such member of Scarabaeidae which causes serious economic damage in sugarcane in tropical India with the potential to inflict 80–100% losses [4]. White grub species belonging to the genus *Holotrichia* are also serious pests in various crops in other parts of the world [5–8]. Available biocontrol measures like application of entomopathogenic fungi and nematodes against *H. serrata* are not satisfactory due to some inherent limitations. For example, entomopathogenic fungi, despite long soil persistence [9], have a long incubation period [10]. The usefulness of entomopathogenic nematodes is limited by non-availability of large-scale mass production techniques [11]. In China, *Holotrichia oblita* and *Holotrichia parallela* are serious pests and several *cry8* genes isolated from *Bt* have been found to possess insecticidal activity against these pests. Genes *cry8Ab1* [12], *cry8Ga1* [13], *cry8Ha1* [14], are toxic to *H. oblita*, whereas *cry8Ab1*, *cry8Ea1* [15], *cry8Ga1*, cry8Ha1, cry8Ia1 [14] and *cry8Na1* [16] are reported to be effective against *H. parallela*. Since no Cry8 proteins are reported till date for *H. serrata,* we prospected for *Bt* isolates carrying scarabid specific *cry8* genes in white grub endemic areas of Tamil Nadu State, India, and identified a *Bt* isolate (*Bt* 62) harbouring a novel *cry8* crystal toxin gene [17]. The novel holotype crystal toxin gene from *Bt* 62 was named *cry8Sa1*and included in the database of the International Committee on *Bt* Toxin Nomenclature, as one of the 28 holotype genes of 60 *cry8* genes reported so far [1]; the sequence information of which was deposited in

* Corresponding author.
E-mail address: singaravelu.b@icar.gov.in (S. B.).
[1] These authors contributed equally to this work.

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GenBank (accession number JQ740599).

Since *Bt* 62 harbours novel *cry8*, it could be potentially used for the management of *H. serrata* either as a *Bt*-based insecticide or by deploying *cry8* genes for development of transgenic plants. The most widely used system for detection of novel crystal toxin genes in *Bt* is traditionally based on polymerase chain reaction (PCR) method [18,19]. Since PCR methods are slow, laborious and time-consuming, and provide only partial sequences, full-length sequences of crystal toxin genes are obtained by construction of a genomic library involving screening and PCR walking strategies [20]. Next-generation sequencing technologies are widely used for the discovery of novel crystal toxin genes which otherwise are not detectable by PCR based methods. Some whole genome sequencing projects of *Bt* harbouring *cry8* genes have revealed that it is not uncommon for the isolates to harbour more than one *cry8* toxin gene [14,21–23]. In order to understand the complete toxin gene spectrum of *Bt* 62 isolate, we undertook its whole genome sequencing using Illumina NextSeq 500 and Oxford Nanopore technology. Further, we characterized the *Bt* 62 isolate for its toxicity, *in vitro* proteolytic activity and histopathological effect in *H. serrata*.

2. Materials and methods

2.1. Genomic DNA extraction

Bt 62 isolated previously by Singaravelu et al. [17] was recovered from glycerol stock stored at −80 °C and grown at 30 °C for 72 h in T3 broth culture. Luria-Bertani (LB) medium and T3 medium were used to extract DNA and parasporal crystals, respectively [24,25]. The parasporal inclusions were examined under a phase-contrast microscope at 1000× magnification from vegetative phase to sporulation. For further examination under scanning electron microscopic (SEM), the spore-crystal mixture was collected, washed twice with sterile deionized water and centrifuged at 6000 rpm for 10 min at 4 °C. The mixture was then re-suspended in sterile deionized water, placed on a glass slide and fixed in 1% OsO_4. The sample was then sputter-coated with gold and platinum for 5 min and SEM micrographs were obtained using a scanning electron microscope FEI Quanta 2000 (Thermo Fisher Scientific Inc., USA) at 20 kV, using the facility available at DRDO Center, Bharathiyar University, Coimbatore, India. Genomic DNA was extracted as described earlier by Reyes-Ramirez and Ibarra [26] with minor modifications and its quality examined by 1% agarose gel electrophoresis. After checking the purity and concentration in NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA), the genomic DNA was stored at −20 °C until further use.

2.2. Illumina library preparation and sequencing

Genome sequencing was performed on Illumina NextSeq 500 platform (Illumina Inc., San Diego, CA) at Bengaluru Genomics Centre Pvt. Ltd. (BGC), Bengaluru, India, using a 150 bp paired-end technology. DNA Illumina library was produced from 1 μg of genomic DNA which was subjected to mechanical shearing using microTUBE AFA Fiber Snap-Cap tubes (Thermo Fisher Scientific, USA) on M220 Focused-Ultrasonicator (Covaris, Woburn, MA, USA). DNA fragments in the range of 250–300 bp length were selected by using the Ampure purification kit (Beckman Coulter Life Sciences, USA) and sequencing libraries were prepared using the Next® Ultra™ II DNA Library Prep kit (New England Biolabs, USA) following the manufacturer's instructions. DNA library was validated using Agilent DNA chip Bioanalyzer (Agilent Technologies, USA) and quantified using QUBIT dS DNA HS Kit (Thermo Fisher Scientific, USA). The raw reads generated using paired-end technologies were further fed to the SPAdes v.3.13.1 assembler [27].

2.3. ONT library preparation and sequencing

MinION Nanopore sequencing was carried out at the *Yaazh Xenomics Pvt Ltd*, Coimbatore, Tamil Nadu, India. The DNA library preparations were conducted as per the protocols provided by the 1D Native barcoding genomic DNA kit with EXP-NBD104, EXP-NBD114, and SQK-LSK109 (Oxford Nanopore Technologies, GA, UK). To construct the library, 1 μg DNA in 48 μl nuclease-free water was randomly sheared in Covaris g-TUBE and centrifuged at 6000 rpm for 60 s to generate fragments of 8 kb average length. The size of fragmented DNA was assessed using the Agilent Bioanalyzer (Agilent Technologies). The DNA nicks were repaired using the FFPE DNA repair mix (M6630, New England Biolabs, NEB, USA). End-repair and dA-tailing were performed using the Ultra II End Prep Module (E7546, NEB, USA) according to the manufacturer's instructions. End-prepped DNA quantification was done using a Qubit fluorometer (Thermo Fisher Scientific, USA) to estimate the final concentration (>700 ng) of DNA prior to Native barcode ligation. The end-prepped DNA was ligated with the native barcodes using the Blunt/TA ligase master mix (M0367, NEB, USA). Further, the resulting barcode sample was ligated with adapters using the NEB Next Quick ligation Module ((E6056, NEB, USA). Purification of ligated product was carried out using 1× Agencourt AMPure XP beads. Prior to loading of prepared library into MinION flow cell, quantification of 1 μl of adapter ligated DNA was done using a Qubit fluorometer (~430 ng) and the MinION flow cell was primed according to the manufacturer's instructions. The final library mix was loaded into the MinION flow cell and sequencing was performed using the MN20826-FLO-MIN106 protocol on the MinKNOW GUI v 2.2. software.

2.4. Genome assembly

The Illumina sequencing reads generated from the paired-end library were subjected to quality screening and trimming using Trimmomatic v.0.39 [28]. The trimming parameters used were ILLUMINACLIP set to 2:30:10 to cut adapter sequences from the read; sliding window 4:15 to check average quality within the window when it falls below threshold; LEADING to cut bases off the start of a read, if below a threshold quality; TRAILING to cut bases off the end of a read, if below a threshold quality; CROP to cut the read to a specified length; HEADCROP to cut the specified number of bases from the start of the read; MINLEN: 36, to drop the read if it is below a specified length; TOPHRED33 to convert quality scores to Phred33 and TOPHRED64 to convert quality scores to Phred-64. Trimmed reads were quality corrected with FASTQ v.0.11.7. The resulting trimmed Illumina reads were assembled into scaffolds using SPAdes v.3.13.1 [27] with k-mer values set to 21, 33, 55 and 77. Assembly module was run using-only the assembler option. Careful option was used to reduce the number of mismatches and short indels [27] for plasmid and short-read assembly. Nanopore reads were extracted, basecalled, demultiplexed, and trimmed using ONT Guppy barcoder v.3.2.2 with the minimum Q score cutoff of 7 (EXP-NBD103) and the quality check was done by NanoPlot v.1.25.0 using summary and loglength options [29]. Passed reads (Q score $\geq$ 7) of length < 2000 bp of ONT runs were assembled using the Canu assembler v.1.8 with corOutCoverage set to 500 with genome size set to 5 m [30]. In addition, the ONT-only long-read assemblies were circularized and trimmed off to remove the overlapped sequence using Circlator v.1.5.5 and Nucmer v.3.1. Circlator v.1.5.5 was used with parameters merge_min_id set to 85 and merge_breaklen set to 1000 [31] and Nucmer v.3.1 tool was used with option 'o' to lists all alignments sorted by the reference coordinates. These Canu long-read assembly consensus were polished using four rounds of Racon polishing v.1.4.0 with default window length of 500, default error threshold of 0.3 and default quality threshold of 10 [32] followed by Medakaconsensus (v.0.8.1,) with option 'm' set to 8 to select the model and to generate the Canu+Polished assemblies. Then *Bt* 62 hybrid assemblies were performed by combining the Illumina short-reads and ONT long-reads in different approaches using HybridSPAdes v.3.13.1 [27] and Unicycler v.0.4.8-beta [33] in which FASTQ files of first and second short reads of the read pair were given using '1' and '2' options respectively. Fasta file of long read was given using 'l' option

[33]. The final hybrid assembly was further polished by 3–4 rounds of Pilon polishing v.1.23 [34] in which 'genome' option was used to give the reference, 'frags' to give fragments of paired end alignments and 'unpaired' option to give unpaired alignments using Plasmid SPAdes reads.

2.5. Genome annotation

The contiguity and quality of the genome assembly were assessed using Quast v.5.0.2 in standard mode [35]. Assembled contigs were aligned and compared with a reference *B. thuringiensis* BT185 genome assembly (GCA_001595725.1) using Mauve tool [36] that distinguishes chromosome, its contiguity, completeness and presence of plasmids. The CDS prediction and genome functional annotation were performed using Prokka software [37]. The predicted CDS were blasted against a reference dataset of insecticidal proteins collected from the online database of *Bt* nomenclature [1] and examined for the presence of Cry, Vip and Cyt insecticidal proteins using Blast2GO v.3.0 [38] with the default blastp and tblastn with the evalue cutoff 1.0E75 and minimum HSP 50. *Bt* toxin scanner (http://bcam.hzaubmb.org/*Bt*Toxin_scanner/) was also used to identify the protoxin composition from predicted coding DNA sequence (CDS) in different contigs. Thus the complete genome features, such as total genome size, number of genes, genes per kbp of the genome and GC % were also investigated.

2.6. Cloning and sequence analysis

PCR amplification of *cry8Sa1* and *cry8Ib* like gene was carried out using two sets of primer pairs from total genomic DNA as template (Supplementary, Table S1). First round PCR amplification was carried out using primers specific to upstream and downstream sequences of both the genes. The second round PCR amplification was done with gene specific primers. For PCR reactions, 50 ng of DNA was added to 20 μl reaction mixture containing 0.25 mM dNTPs, 0.3 μM each of the forward and reverse primers, 2.0 μl of 10× buffer, 0.5 μM $MgSO_4$ and 1 U of Platinum Taq DNA polymerase (Invitrogen, USA). PCR reaction was performed in an Eppendorf Master-cycler and amplification conditions were as follows: an initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s and extension at 68 °C for 4 min, and a final extension step at 68 °C for 10 min. Amplified products were analysed on 1% agarose gel electrophoresis, gel purified using Qiagen gel extraction kit and the concentration was quantified using Nanodrop (Thermo Fisher Scientific, USA). The purified PCR product was ligated into the cloning vector pGEMT EASY (Thermo Fisher Scientific, USA) using T4 DNA ligase (NEB, USA) and transformed into *E. coli* DH5α competent cells grown under ampicillin selection marker. Recombinant clones were screened using colony PCR and confirmed by Sanger sequencing. The gene sequences were subjected to BLAST against the nucleotide and protein databases of NCBI. Further, the endotoxin domains were determined in comparison with closely related Cry8 proteins by using the CDvist tool with default parameters [39] (http://cdvist.zhulinlab.org/). CLC Main Workbench v. 7.9.1 was used to deduce and compare the amino acid sequences of *Bt* 62 Cry8Sa and Cry8Ib with the closely related Cry8 proteins. Cry8Sa and Cry8Ib protein structures were modelled using Phyre2 web server (http://www.sbg.bio.ic.ac.uk/phyre2.) under the intensive mode [40].

2.7. Phylogenetic analysis of Bt 62 genome and Cry8 proteins

The phylogenetic relationship of *Bt* 62 was constructed using the alignment-free feature frequency profile (FFP) method [41]. The whole genome sequences of 48 *Bt* isolates were retrieved from NCBI and converted into an RY (purine/pyrimidine)-coded form (FFPry) to calculate the l-mer frequency profiles of each genome. The utilities *ffpvprof* and *ffpreprof* were used to estimate the lower and upper word limit of the l-mers to be used for FFP calculations. The optimal feature length was determined using the convergence method and l-24 was chosen for the analysis. The phylogenetic tree was constructed using 1000 replicated neighbor-joining methods, based on a Jensen-Shannon divergence distance matrix calculated from the normalized feature frequency profiles. Subsequently, the majority consensus tree was obtained using the CONSENSE utility of the PHYLIP package. Similarly, the phylogeny for all the *cry8* holotypes was constructed using MEGA version X [42]. Amino acid sequences from all the Cry8 proteins listed in Bacterial Pesticidal Protein Resource Center (BPPRC) were retrieved from NCBI database [1] and multiple sequence comparison was carried out with Log-Expectation (MuSCLE) program [43] in MEGA X [42]. Gap penalties were set with gap open to −2.9, gap extends to 0 and hydrophobicity multiplier to 1.2. UPGMB (Unweighted Pair Group Method using Arithmetic Mean) clustering method, a variant of UPGMA was used. Minimum diagonal length (lambda) was set at 24 and maximum memory iterations were set at default value of 16. The phylogenetic tree was constructed using the Neighbor-Joining method with amino acid substitution type and Jones-Taylor-Thornton (JTT) model. Uniform rates were set for defining substitution rates among sites. ML (Maximum-Likelihood) Heuristic Method used was Nearest-Neighbor-Interchange (NII). Number of threads was set to 3. Gaps/missing data treatment was given using the complete deletion method. The number of bootstrap replications was set to 1000.

2.8. Total cell protein isolation and protein profile analysis

The protein profile of *Bt* 62 was studied by SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the discontinuous system [44]. Overnight grown culture was streaked on a fresh T3 medium plate and incubated at 30 °C for 72 h. The culture was then scraped off the plate by adding 5 ml of sterile double distilled water. The spore-crystal mixture was collected by centrifugation at 7000 rpm and 4 °C for 10 min and the pellet was re-suspended in sterile ice-cold distilled water. The suspension was centrifuged and washed twice with ice-cold sterile distilled water. The mixture was vortexed thoroughly and sonicated for 1 min twice at 1 min interval. The total protein concentration in the spore crystal mixture was estimated as per the method described by Bradford, [45] using Bovine Serum Albumin as standard. 20 μl (200 μg) of spore crystal mixture was loaded in each well of 10% SDS-PAGE gel electrophoresis and the protein profile was analyzed after staining the gel with 0.1% Coomassie Blue R-250.

2.9. Proteolysis assay

The purification of crystal protein from the spore crystal mixture was done as per an earlier procedure [46,47]. Spores and crystals were harvested by centrifugation, and washed thrice with NaCl (1 M) and three times with sterile ice-cold double-distilled water. The spore-crystal mixtures were then solubilized in 50 mM sodium carbonate buffer (pH 9.5) and the mixture was subjected to 1 min sonication thrice at 2 min intervals and then the protein mixture was incubated at 37 °C for 2 h at 80 rpm. After centrifugation at 20,000 rpm for 10 min, the purified delta-endotoxins were recovered in soluble fraction and analyzed by SDS-PAGE. For the extraction of midgut juice of white grub, third instar larvae were dissected and midgut was isolated. The gut juice was then extracted after grinding the tissue with 0.1 M NaCl. The concentrations of midgut protein and bacterial crystal fractions were determined by Bradford assay using bovine serum albumin as a standard [45]. *In vitro* proteolytic assay was carried out by incubating midgut protein (10 μg) and protoxins (200 μg) in a ratio of 1: 20 (*w*/w). Reaction mixtures were incubated at room temperature with constant agitation for 30 min, 1 h, 3 h, 6 h, 12 h, 16 h and 24 h. The proteolysis reaction was stopped by the addition of 0.1 mM phenyl methyl sulphonate (PMSF) final concentration. Proteolysis products were separated on 10 % SDS-PAGE.

2.10. Insect bioassay

A laboratory culture of *H. serrata* was maintained during June–September from field collected white grub adults [11]. Bioassay with *Bt* 62 toxin was conducted against first instar grubs using washed spore crystal mixture by carrot disc contamination method. Freshly peeled carrots were first cut in to 2–3 mm thick discs of 30 mm dia. which were further sized to 10 mm dia. discs using a microbiological cork borer. Ten µl of diluted toxin suspension was dispensed on one side of the disc to give effective dosages of 1, 2.5, 5.0, 7.5 and 10 µg per disc; carrot discs treated with sterile water served as control. Treated discs were air dried before provisioning one disc per grub in individual wells of 12-well tissue culture plates. Three replications of five grubs each were maintained for both treatment and control and mortality of grubs was recorded at 24 h intervals. Data of mortality in first instar were subjected to arcsin or square root transformation [48] and repeated measures ANOVA, with posthoc comparison of means by Tukey HSD test [49], to determine the effect of dosage, duration of exposure and their interaction.

2.11. Histopathological studies of midgut

The histopathological effect of *Bt* 62 protein on the midgut of *H. serrata* was studied in first instar larvae with a slight modification of an earlier protocol used for a lepidopteran insect [47,50]. Grubs fed with carrot discs contaminated with protoxin of *Bt* 62 were dissected two days after exposure and midguts collected; midguts of grubs fed with plain carrot discs served as control. The dissected midguts were washed with 0.1 M phosphate-buffered saline (PBS, pH 7.4), and the tissues were dehydrated in ethanol gradient and embedded in paraffin. Ultrathin sections of 5 µm were prepared using a rotary microtome (Leica Biosystems, Germany) and placed in carriers coated with egg albumin and 10% formaldehyde. Subsequently, the sections were deparaffinized in three changes of xylene for 2 min each, rehydrated in ethanol series, rinsed in running tap water and stained with hematoxylin (nuclear stain) and eosin (cytoplasmic stain). The sections were then dehydrated in two changes of 95% and 100% ethanol, cleared in xylene and mounted using DPX mountant. Images were observed and photographed using a light microscope (Zeiss Micro. LTD, Germany).

3. Results

3.1. Crystal morphology

Crystalline inclusions of *Bt* 62 appeared spherical under a phase-contrast microscope (Fig. 1A). Ultra-structural analysis of the spore/crystal mixture by SEM showed the spherical crystals and spores distinctly (Fig. 1B).

3.2. Whole genome sequencing of Bt 62

Whole genome sequencing of *Bt* 62 was carried out using the combination of Illumina NextSeq500 and Oxford Nanopore Technology (ONT) sequencing platforms (Supplementary Fig. S1). In order to achieve complete genome assembly, we carried out hybrid assembly of Illumina and Nanopore reads using five different assemblers, namely SPAdes, Canu, Canu Polished, Hybrid SPAdes and Unicycle. The detailed analysis report has been illustrated in Table1. Among the five assemblies described above, the long read based Canu polished assembly and the Unicycler hybrid assembly resulted in larger and contiguous contigs than the rest of the assemblies. Hybrid assembly with SPAdes produced 65 contigs while Canu polished and Unicycler gave five contigs. Finally, the complete genome was deduced from ONT (Canu polished) assembly followed by polishing with Illumina reads rather than data from the Hybrid assembly alone (Table 1). In order to determine the overall completeness and genome organization of *Bt* 62 genome assembly, we performed reference based genome assembly and compared with the *Bacillus thuringeinsis* strain *Bt*185 genome using progressive- Mauve tool. Mauve alignment indicated the completeness of the *Bt* 62 genome assembly build and showed that the organization of the LCB (Locally Collinear Blocks, each differently colored blocks) were much conserved between the chromosome of the two strains. However, there is very less similarity among the plasmids carried by both the strains (Supplementary Fig. S2). The complete assembly of *Bt* 62 genome contains one circular chromosome of 5,294,964 bp with an average GC content of 35.3% and four plasmids, *viz.* pBT62A of length 561,791 bp with 33.4% GC, pBT62B of 262,775 bp with 32.9% GC, pBT62C of 10,512 bp with 30.8% GC and pBT62D of 3239 bp with 34.4% GC (Fig. 2 and Table 2). Further analysis of genomic sequences identified 6269 genes, of which two are insecticidal protein coding DNA sequences (CDS), 107 tRNAs and 42 rRNAs (Table 2). The annotated genome of *B. thuringiensis* strain *Bt* 62 was deposited in the NCBI under the Bioproject with accession number PRJNA427501.

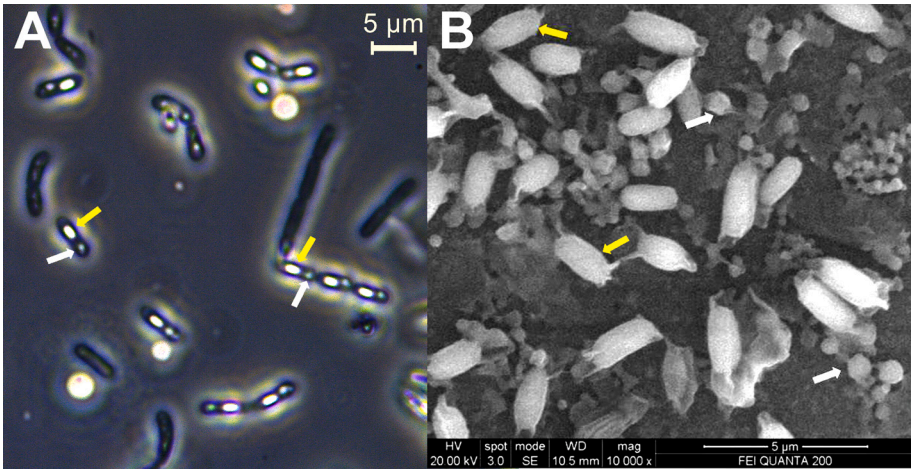

Fig. 1. (A) Phase-contrast micrograph of *Bt* 62 isolate showing spores and crystal toxin. (B) Scanning electron micrograph of autolyzed endospores and spherical parasporal crystals; arrows indicate spore (yellow) and crystal (white). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Cry genes identification

When the protoxin composition of the predicted CDS was analyzed, two full length *cry* genes, namely c*ry8Sa1* and c*ry8Ib* like and a partial *cry* gene *cry8Ma3* were identified. Analysis of *cry8Sa1* and c*ry8Ib* like genes revealed that both the genes are located in the plasmid pBT62B corresponding to 198,489–202,085 bp for c*ry8Sa1* gene (3597 bp), and 205,075 bp to 208,653 bp for c*ry8Ib* like gene (3579 bp). However, in the same plasmid *cry8Ma* like partial sequence was identified in the regions 212,314 to 214,029 bp, 214,060 to 214,350 bp and 214,060 to 214,350 bp. Thus the whole genome analysis of *Bt* 62 identified *cry8Sa1* and *cry8Ib* like crystal toxin genes which are predicted to encode for Cry8 proteins. In order to characterize the gene sequences, we cloned and sequenced the full length genes and analyzed for crystal toxin protein characteristics (Supplementary Table S2, Supplementary Figs. S3, S4 and S5). BlastP analysis of deduced amino acid sequences of Cry8Sa1 revealed its identity to Cry8Ib3 endotoxin protein (AHG25076.1) with a similarity of only 62.81% which designates Cry8Sa1 as a novel holotype. This is the first novel *cry8* holotype gene isolated from India. Cry8Ib like gene present in the *Bt* 62 isolate encodes a toxin identical to Cry8Ib2 delta endotoxin (AGU13842.1) with a similarity of 94.62%. Conserved domain analysis of deduced amino acid sequences of *Bt* 62 Cry8Sa1 and Cry8Ib like proteins revealed that both proteins have Crystal toxin domains similar to other Cry8 proteins. There are four positions of endotoxin domains, namely endotoxin_N, endotoxin_M and two endotoxin_C observed in all the Cry8 proteins. Normally, endotoxin C domain is located in the region between 500 aa −700 aa of Cry toxins. However, the position of one of the endotoxin_C domains (737 aa - 879 aa) in Cry8Sa1 was found to be unique when compared with other Cry 8 proteins (Supplementary Table S3). Whereas, *Bt* 62 Cry8Ib like protein is carrying an extra endotoxin_C domain (1098 aa - 1193 aa) like Cry8Ia (1097 aa - 1192 aa) when compared to other Cry8 proteins (Supplementary Fig. S6). Comparison of deduced amino acid sequences of Cry8Sa1 and Cry8Ib like proteins from *Bt* 62 with the closely related Cry8 proteins revealed eight conserved blocks. In *Bt* 62 CrySa1, the conserved blocks are present in the region 184 aa – 215 aa (Block I); 257 aa – 312 aa (Block II); 493 aa-539 aa (Block III); 563 aa – 573 aa (Block IV); 638 aa – 647 aa (Block V); 673 aa −729 aa (Block VI); 773 aa – 806 aa (Block VII) and 866 aa – 911 aa (Block VIII) whereas in *Bt* 62 CryIb, the conserved blocks are present in the region 191 aa – 220 aa (Block I); 269 aa – 319 aa (Block II); 509 aa – 555 aa (Block III); 580 aa – 590 aa (Block IV); 655 aa – 664 aa (Block V); 692 aa – 746 aa (Block VI); 788 aa – 821 aa (Block VII) and 881 aa – 926 aa (Block VIII) (Supplementary Fig. S7). Structural modelling of Cry8Sa1 and Cry8Ib proteins from *Bt* 62 using the known Cry1Ac protein structure (PDB code: 4w8j) revealed seven conserved domains in the region of 14 aa – 281 aa (Domain I), 297 aa – 515 aa (Domain II), 521 aa – 648 aa (Domain III), 650 aa – 721 aa (Domain IV), 759 aa – 910 aa (Domain V) and 913 aa-1015 aa (Domain VI) for both the proteins. But the position of Domain VII was 1056 aa - 1195aa for Cry8Sa1 and 1056 aa - 1188 aa for Cry8Ib (Supplementary Fig. S8).

3.4. Phylogeny

The phylogenetic tree inferred from the whole genome data of 48 taxa is presented in Fig. 3A. Phylogenetic analysis showed that association of *Bt* 62 with other completely sequenced *B. thuringiensis* genomes aligns in the clade with the highest bootstrap value and clustered within

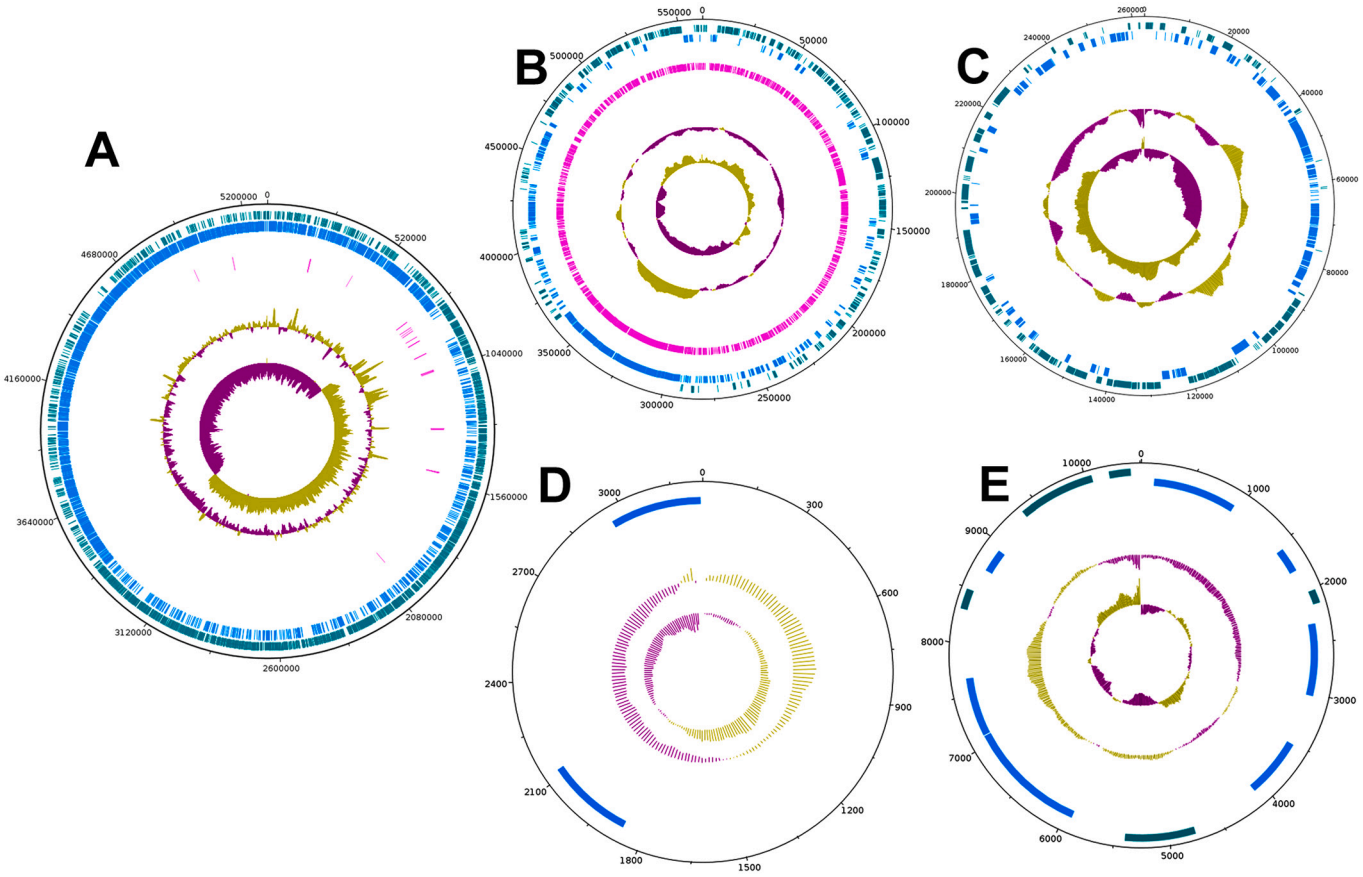

Fig. 2. Schematic representation of *Bt* 62 genome: Circular map of chromosome (A) and plasmids (B, C, D and E). The outer green circle denotes genome alignment between *Bt* 62 and the reference genome. Blue bars denote coding sequences, pink bars denote functional genes, inner circles 1 and 2 showing GC skew (circle 1) and GC plot (circle 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparative assembly statistics of *Bt* 62 genome.

Assembler	SPAdes	Canu	Canu polished	Hybrid spades	Unicycler
Number of contigs retrieved	399	8	5	65	5
Number of contigs <2000 bp	296	2	nil	44	nil
Total genome size (bp)	6,004,043	6,175,969	6,133,281	6,123,896	6,126,052
Assembly GC (%)	34.8	35.11	35.03	34.96	35.01
Reference GC (%)	35.42	35.42	35.42	35.42	35.42
N50	163,127	5,255,988	5,294,964	9564,29	5,289,233

a subgroup, comprising *B. thuringiensis* serovar alesti BGSC4C1, *B. thuringiensis* MYBT18246, *B. thuringiensis* BT-59, and *B. thuringiensis* HD-771. However, comparison of plasmids within the same phylogenetic cluster did not reveal any similarity for *Bt* 62 plasmids. Further, Cry8Sa1 and Cry8Ib like proteins were analyzed for phylogeny with other 24 Cry8 holotypes recognized by the international committee on *Bt* nomenclature (Fig. 3B). The results revealed that all the Cry 8 proteins formed three main groups. In group I, Cry8Bb, Cry8Bc, Cry8Ba, Cry8Pa, Cry8Ea, Cry8Fa, Cry8Qa, Cry8Ka, Cry8Kb, Cry8Aa, and Cry8Ab are closely related and clustered in different clades. In group II, Cry8Da and Cry8Db clustered within the same clade and grouped with Cry8Ca, Cry8Ja, Cry8Ga, and Cry8Na proteins. In group III, Cry8Hb and Cry8Ra are closely related to each other and clustered within the same clade. A similar phenomenon was also observed with Cry8La and Cry8Ma. *Bt* 62 Cry8Sa is closely related to the Cry8La and Cry8Ma whereas *Bt* 62 Cry8Ib like protein grouped within a clade of already reported Cry8Ib and closely related to Cry8Ia (Fig. 3B).

Table 2
Genome features of *Bt* 62 Hybrid assembly.

Feature	Length (bp)	GC (%)	No. of CDS	Total predicted gene length (bp)	No. of genes	No. of tRNAs	No. of rRNAs
BT62 chromosome	5,294,964	35.3	5318	4,500,964	5467	107	42
Plasmid pBT62A	561,791	33.4	515	407,592	515	–	–
Plasmid pBT62B	262,774	32.9	271	187,647	271	–	–
Plasmid pBT62C	10,512	30.8	13	6223	14	–	–
Plasmid pBT62D	3239	34.4	2	537	2	–	–

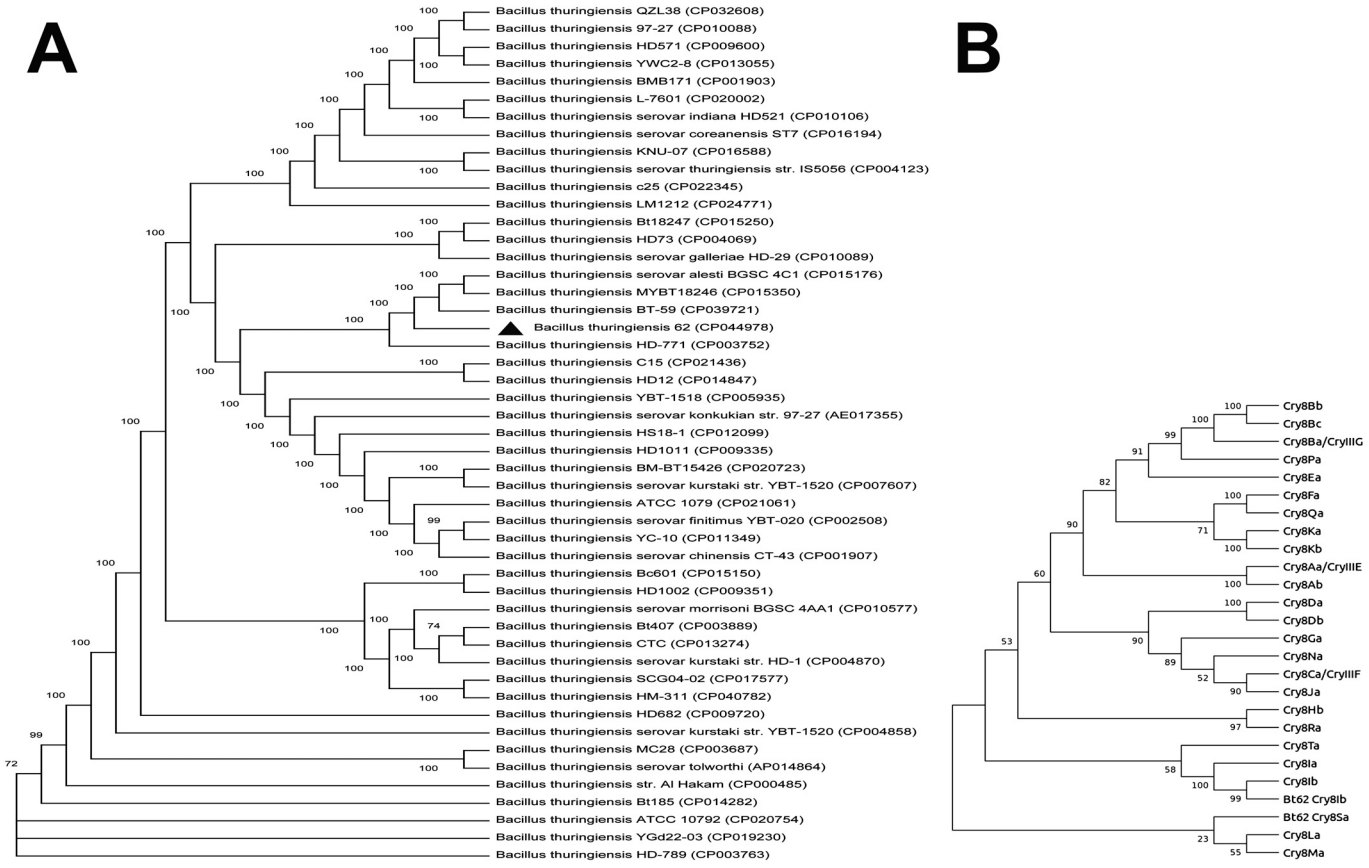

Fig. 3. (A) Phylogenetic analysis of *Bt* 62 genome with other *Bacillus thuringiensis* genome sequences (48 isolates published in NCBI) using FFP method with 1000 bootstrap replications. (B) Phylogenetic analysis of Cry8 proteins listed in Bacterial Pesticidal Protein Resource Center (BPPRC) were retrieved from the NCBI database and aligned using Multiple Sequence comparison by Log-Expectation (MuSCLE) program. The maximum-likelihood (ML) tree was constructed using MEGAX and bootstrap confidence values were generated using 1000 permutations.

3.5. Protein profile and proteolytic activity

The protein profile of *Bt* 62 showed a prominent band of approximately 135 kDa size indicating likely presence of both Cry8Sa1 and Cry8Ib like proteins (Fig. 4A). Results of the proteolytic assay showed the release of about 65 kDa, a functional form of crystal protein, as early as one hour after incubation which continued up to 16 h. As the proteolytic activation progressed, the concentration of native band of crystal protein (135 kDa) decreased with incubation time whereas the active form of 65 kDa protein seemed to become more intense (Fig. 4B).

3.6. Bioassay against H. serrata

At a dosage range of 1.0–10.0 µg/disc, *Bt* 62 caused dosage-dependent mortality in first instar grubs of *H. serrata* 1 day after treatment (DAT) and mortality rates increased progressively with time of exposure (Table 3). Repeated measures ANOVA of mortality was significant for dosage ($F = 12.11$; df = 5, 12; $P < 0.001$) and days after treatment (DAT) ($F = 14.80$; df = 2, 24; $P < 0.0001$) but not their interaction ($F = 0.99$; df = 10, 24 $P > 0.05$). Mean mortality rates at different dosages were considerably high but did not differ among themselves; however, they were significantly higher than that in control. Such non-significant differences indicated that first instar grubs were susceptible to the toxin even at lower dosages used. Mortality rates observed 1 DAT, indicative of acute toxicity, increased significantly to reach a peak 2 DAT but remained the same up to 5 DAT. Such time-dependent mortality suggested chronic toxic effect upon continuous exposure to the toxin (Table 3 and Fig. 5).

3.7. Histopathological effect

Histopathological observations of *H. serrata* larvae indicated extreme membrane perturbation in the midgut 24 h after treatment (Fig. 6). The midgut of treated and control grubs showed drastic differences in the delicate epithelial tissues suffering intensive damage with the debris clearly visible in the lumen (Fig. 6B and D). No recognizable changes were observed in the midgut of the control larvae which had well-defined epithelial cells with intact lumen and midgut wall (Fig. 6A and C). Also, while the control larvae showed undisturbed lumen lined with numerous normal microvilli (Fig. 6C), in the *Bt* 62 treated larvae the undistinguished apical brush border was no longer continuous and microvilli were disrupted.

Table 3
Bioassay of *Bt* 62 against first instar grubs of *Holotrichia serrata* by carrot disc feeding method.

Dose (µg/disc)	Cumulative grub mortality (%) at different intervals (d)			
	1 DAT	2 DAT	5 DAT	Mean[#*]
1.0	53.3 ± 11.5	66.7 ± 11.5	73.3 ± 23.1	64.4 (55.1) a
2.5	60.0 ± 20.0	80.0 ± 34.6	93.3 ± 11.5	77.8 (67.9) a
5.0	80.0 ± 20.0	93.3 ± 11.5	93.3 ± 11.5	88.9 (76.1) a
7.5	80.0 ± 20.0	93.3 ± 11.5	100.0 ± 0.0	91.1 (78.9) a
10.0	73.3 ± 11.5	86.7 ± 23.1	100.0 ± 0.0	86.7 (74.7) a
0	6.7 ± 11.5	6.7 ± 11.5	6.7 ± 11.5	6.7 (9.7) b
Mean[#*]	58.9 (50.4) A	71.1 (62.3) B	77.8 (68.5) B	

[#] Figures in parentheses are arcsin transformed values.
[*] Means separated by the same lower case letter in the column and upper case letter in the row are not significantly different ($P > 0.05$) by repeated measures ANOVA and Tukey HSD test.

4. Discussion

Bacillus thuringiensis has been used as an efficient biopesticide in agriculture due to its ability to reduce pest populations [51]. The aim of the present study was to understand the complete toxin gene spectrum of *Bt* 62 isolate and its functional characterization against *H. serrata*, a serious pest of many field crops including sugarcane wherein it can potentially cause up to 100% damage. Our previous studies with *Bt* 62 isolate revealed a novel crystal toxin gene *cry8Sa1* [17]. The whole genome sequencing of the isolate carried out in the present study led to the identification of another *cry8* gene which shares 94.62% amino acid identity with *cry8Ib2* [1]. The presence of multiple *cry8* genes, in addition to other classes of *Bt* toxin, has been reported previously in the *Bt* strain HBF-18 [21]. This isolate was originally discovered to encode *cry8G* [13] but whole genome sequencing revealed the presence of three additional and novel genes, namely *vip1Ad1, vip2Ag1* and *cry8*-like in this strain. The whole genome sequencing of another *cry8 Bt* strain INTA Fr7-4 [23] revealed only the presence of the reported insecticidal genes *cry8Kb3*, *cry8Pa3,* and *cry8Qa2* along with two *vip2-vip1* operons [52–53]. Similarly, another strain *Bt* 185 which was reported to encode Cry8Ea1 and Cry8Fa1 earlier was observed to harbour a new *cry8Ab*-like gene [22]. Likewise, genome sequencing of *Bt*SU4, a coleopteran active

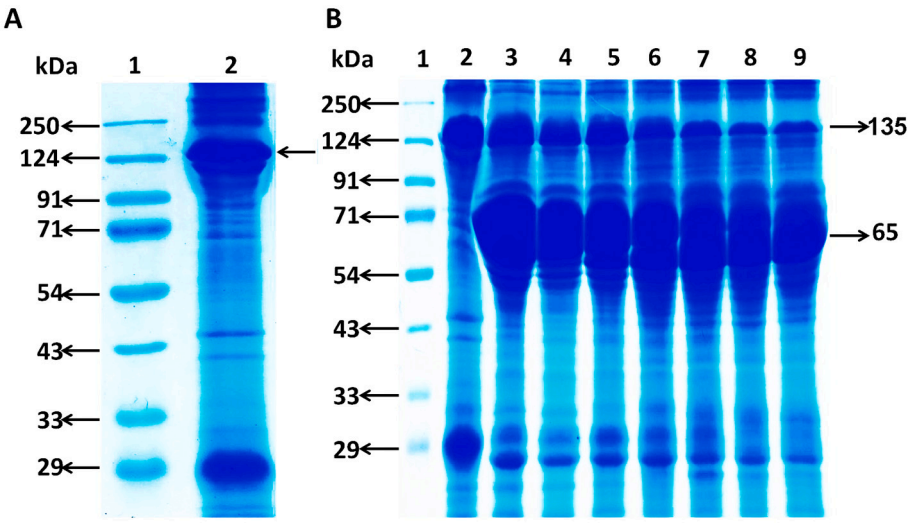

Fig. 4. SDS-PAGE analysis of *Bt* 62 Cry protein (A) Protein profile of *Bt* 62 spore-crystal mixture: Lane 1 - molecular mass markers in the range of 10–250 kDa; Lane 2–135 kDa protoxins Cry8Sa1 and Cry8Ib indicated by an arrow. (B) *In vitro* time-course proteolytic activation of *Bt* 62 protoxins by *Holotrichia serrata* midgut proteases: Lane 1 - molecular weight marker (10–250 kDa); Lane 2 - *Bt* 62 protoxins; Lane 3 to 9 - *Bt* 62 activated toxins incubated with midgut proteases for 30 min, 1 h, 3 h, 6 h, 12 h,16 h and 24 h, respectively. Arrows indicate protoxin (135 kDa) and activated toxin (65 kDa).

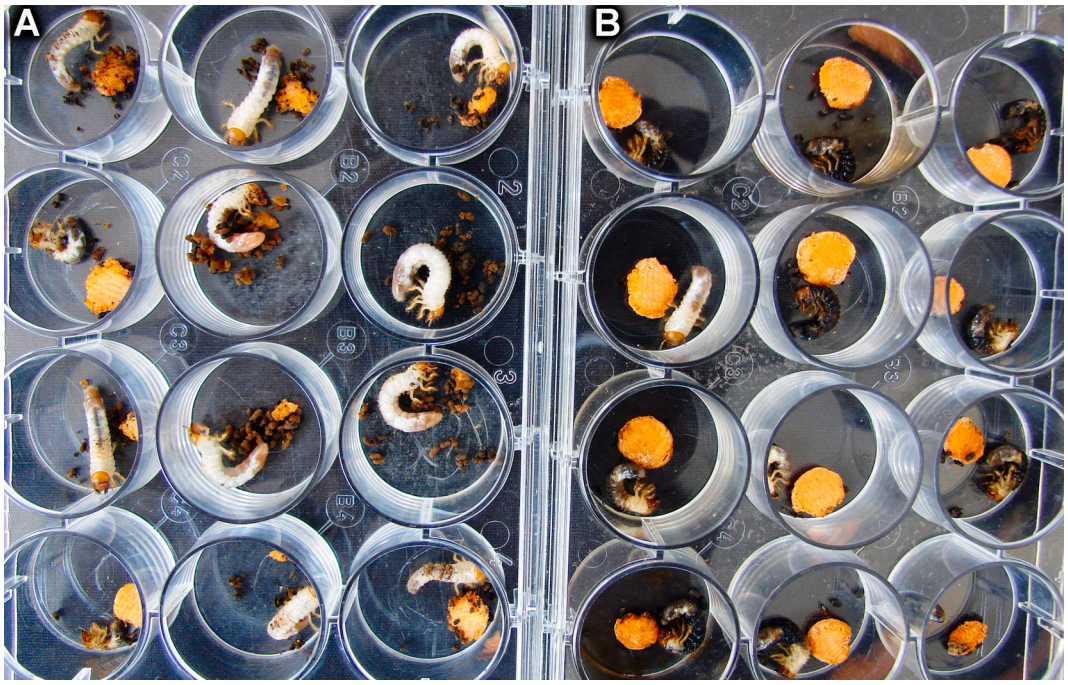

Fig. 5. Bioassay of *Bt* 62 mixture against 1st instar grubs of *Holotrichia serrata.* (A) grubs released on carrot discs treated with sterile water as control (B) grubs exposed to carrot discs contaminated with spore-crystal mixture of *Bt* 62 isolate.

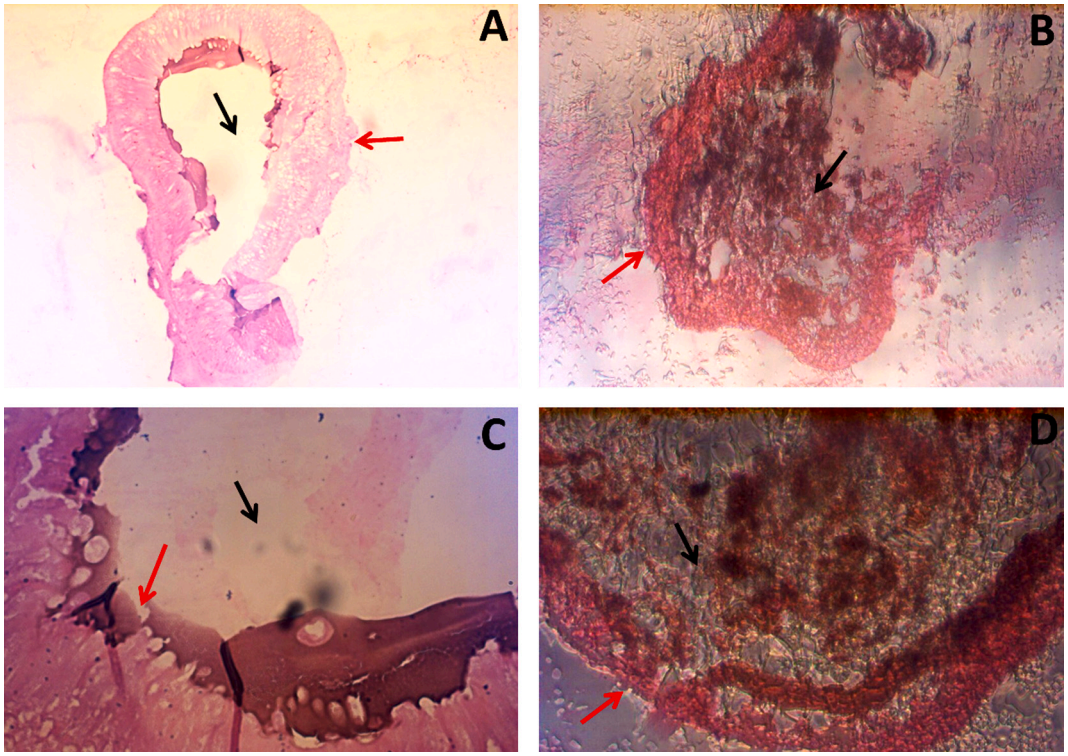

Fig. 6. Histopathological effects of *Bt* 62 toxin on midgut of *Holotrichia serrata* grubs; cross section of midgut showing intact tissue in untreated grubs (A and C); cross section of midgut showing degenerated tissue in *Bt* 62 treated grubs (B and D). Red and black arrows correspond to midgut wall and lumen respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isolate, revealed two new cry8 genes, designated as *cry8Ha1* and *cry8Ia1* [14]. *Bt* 62 was found to contain only *cry8* genes in contrast to other Cry8 producing *Bt* strains described above that are known to encode Vip1 and Vip2 proteins also. Similarly, there is variation in plasmid number between *Bt* 62 and other *cry8 Bt* isolates studied. While *Bt* 62 carried four plasmids like INTA Fr7-4 [23], the strain *Bt* 185 was found to carry eight plasmids [22]. The two complete *cry8* gene sequences of *Bt* 62, i.e. *cry8Sa1* and *cry8Ib* like toxin gene were present in the same plasmid, a feature similar to other *cry8 Bts* described above.

Cry8-type toxins have been described as toxic to coleopteran pests,

mainly members of the Scarabaeidae family [54]. For example, a novel *cry8Ga1* gene encoding a toxin was identified and cloned from HBF-18 *Bt* strain, whose toxicity was established against *Holotrichia parallela* larvae [21]. In our study, when spore crystal suspension of *Bt* 62 containing two Cry8 proteins was fed *per os* to first instar *H. serrata* grubs, we observed mortality one DAT. Progressive increase in mortality levels suggested chronic toxic effect upon continuous feeding on contaminated carrot discs. The general dosage-dependent mortality indicated that first instar grubs are susceptible to the toxins produced by the *Bt* 62 isolate. Since the spore crystal suspension of *Bt* 62 used in the bioassay contains two Cry8 toxins, it is essential to ascertain which of these two toxins is effective against *H. serrata*. The most common approach for identification of functional toxicity of individual Cry proteins present in the same *Bt* isolate is through expression of *cry* genes in acrystalliferous *Bt* strains using shuttle expression vector system. Individual expression of *cry8Kb3*, *cry8Pa3* and *cry8Qa2* present in the *Bt* strain INTA Fr7-4 in acrystalliferous *Bt* revealed that only Cry8Pa3 showed toxicity against *Anthonomus grandis* larvae among the three recombinant toxins tested [52–53]. When the two novel *cry8*-type genes, namely *cry8Ea1* and *cry8Fa1* present in the *Bt* strain *Bt*185 were expressed in acrystalliferous mutant strain HD73^{-}, *cry8Ea1* alone was toxic to *Holotrichia parallela* [15]. Similarly, some other *cry8* genes from various *Bt* strains were expressed in acrystalliferous *Bt* strains and toxicity to *Holotrichia* genus determined [13,15,54].

The first step in the molecular characterization of *Bt* is to study crystal morphology which includes bipyramidal, cuboidal, rectangular and spherical shapes as this can provide valuable information on target insect spectra [55]. Scanning electron microscopy of the parasporal crystals produced by *Bt* 62 revealed them as spherical shaped. Similarly, spherical crystals were produced by the scarabaeid specific strain Buibui of the serovar *japonensis* expressing Cry8Ca proteins [56,57]. It is a well-established fact that *cry8* genes encode proteins of 130 to 135 kDa size which are active against coleopteran pests [13,15]. *Bt* 62 showed a distinct protein band of 135 kDa which indicated the presence of Cry8 proteins. Consequently, sequence analysis of two full length genes, namely *cry8Sa1* and *cry8Ib* like gene and the corresponding amino acid sequences deduced a molecular mass of 136.17 and 135.24 kDa, respectively.

Similar to other Cry8 proteins, *Bt* 62 Cry8Sa1 and Cry8Ib revealed the presence of eight conserved blocks which are known to occur in Cry proteins [15]. We also observed the five conserved blocks of the toxic core to be present within the three-domain region as found in other Cry proteins [58].

Bt crystal protoxins are activated in the target insect by proteolytic activity of insect midgut proteases as full-length unprocessed crystal toxin is incapable of forming pores *in vivo* which is critical to their mode of action [59]. Our results revealed that the reaction containing midgut juice of *H. serrata* and *Bt* 62 toxin produced ~65 kDa active toxin as described earlier for Cry8Ca2 [60]. This indicated clearly that protease activity is involved in *H. serrata* larvae for the activation of Cry8 protein. Additionally, the interaction of Cry toxin with midgut proteins is an important process in the mode of action of Cry toxins [58]. The examination of histopathological effect of *Bt* 62 Cry toxin in *H. serrata* demonstrated that active toxin disrupted epithelial cells. Such histopathological alterations were observed in other coleopteran insects too [61]. The consequent leakage of material into the lumen would eventually have led to the death of the intoxicated insects in our bioassays.

Although *Bt* isolates toxic to *H. serrata* were reported earlier [62], the isolates were not characterized for their crystal toxin gene content and evidence for their toxicity was not demonstrated by *in vitro* proteolytic studies. The present proteolytic studies for our *Bt* 62 isolate clearly demonstrated activation of its protoxin to an active toxin core of ~65 kDa. This is further supported by the presence of two scarabid specific *cry8* genes making our isolate the first report of authentic identification of *Bt* toxic to the white grub *H. serrata*. Besides, of the two *cry8* genes in *Bt* 62 isolate, *cry8Sa1* was recognized as a holotype crystal toxin gene by the International Committee on *Bt* Toxin Nomenclature [1]. Deployment of these crystal toxin genes for developing transgenic crops for white grub resistance needs to be explored. Earlier, genetically engineered peanut and soybean expressing *cry8Ea1* and *cry8* like gene were found effective against *H. parallela* [63,64]. The underground nature and insidious attack by *H. serrata* render it difficult to discern initial damage for timely implementation of control measures. However, when the grubs reach third instar and damage becomes severe, control measures become less effective. From the management perspective, genetically modified crops expressing the toxin can give innate resistance to first instar grubs preventing their development to later stages that are voracious feeders.

5. Conclusions

In the present study, we characterized a novel coleopteran specific *B. thuringiensis* isolate *Bt* 62. Whole genome sequencing of the isolate revealed two Cry8 protein coding genes, namely *cry8Sa1* and *cry8Ib* like gene. We also demonstrated the functional toxicity of *Bt* 62 isolate against the white grub *H. serrata*, a serious pest of sugarcane. *In vitro* proteolytic assay and histopathological effect of *Bt* 62 on *H. serrata* added more evidence of the functional toxicity of the Cry8 toxins expressed in the isolate. Many holotype *cry8* genes have been described in literature against various *Holotrichia* species but this is the first report of an *H. serrata* toxic *Bt* isolate which harbours a novel *cry8* holotype gene *cry8Sa1*. The new *Bt* isolate characterized in the present study could serve as a potential source of candidate genes for developing transgenic crops resistant to *H. serrata.* However, functional toxicity of each of the two genes, alone or in combination, needs to be established for their use in developing transgenic sugarcane and other crop. Alternatively, until such information is generated and transgenics are developed, *Bt* 62 could be used as an effective bio-pesticide to manage white grub in sugarcane and other crops.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.12.012.

Data availability statement

The dataset produced in the present work have been deposited in the NCBI under the accession numbers PRJNA427501, SAMN08238639, GCA_003054785.1, GCA_003054785.2, CP044978.1, CP044979.1, CP044980.1, CP044981.1 and CP044982.1.

Author contributions

BS conceptualized and designed the study; BS, JS, KH, CS and RN are involved in screening and isolation of *Bt* strain; GSS, BS, AC, MN and PCS are involved in whole genome sequencing and data analysis; MN and GSS have carried out experiments on proteolytic assay and histopathological studies; BS, JS, RN and PM have conducted bioassay experiments; GSS, BS, JS and MN are involved in drafting, editing and revising the manuscript; NC and BR provided guidance for the study.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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